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Evidence for Electrogenic Sodium-Dependent Ascorbate Transport in Rat Astroglia

John X. Wilson[1], Ewa M. Jaworski[1], and S. Jeffrey Dixon[1,2]

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The dependence of ascorbate uptake on external cations was studied in primary cultures of rat cerebral astrocytes. Initial rates of ascorbate uptake were diminished by lowering the external concentrations of either Ca^{2+} or Na^+. The Na^+-dependence of astroglial ascorbate uptake gave Hill coefficients of approximately 2, consistent with a Na^+-ascorbate cotransport system having stoichiometry of 2 Na^+:1 ascorbate anion. Raising external K^+ concentration incrementally from 5.4 to 100 mM, so as to depolarize the plasma membrane, decreased the initial rate of ascorbate uptake, with the degree of inhibition depending on the level of K^+. The depolarizing ionophores gramicidin and nystatin slowed ascorbate uptake by astrocytes incubated in 5.4 mM K^+; whereas, the nondepolarizing ionophore valinomycin did not. Qualitatively similar results were obtained whether or not astrocytes were pretreated with dibutyryl cyclic AMP (0.25 mM for 2 weeks) to induce stellation. These data are consistent with the existence of an electrogenic Na^+-ascorbate cotransport system through which the rate of ascorbate uptake is modulated by endogenous agents, such as K^+, that alter astroglial membrane potential.

KEY WORDS: Ascorbate; astrocytes; membrane potential; transport mechanism; rat brain; Vitamin C.

INTRODUCTION

Ascorbate (reduced vitamin C) influences brain function because it modulates the production of myelin (1,2) and the biosynthesis, release, receptor-binding and clearance of transmitters (4–12). The mental depression which occurs as an early symptom of scurvy (13) suggests there is an important physiological role for neuromodulation by normal levels of ascorbate in brain. The molecular mechanism of action of ascorbate appears to derive from its activity as an antioxidant and reducing agent (14).

Ascorbate is not synthesized by brain cells (15) and is not detectable in either astrocytes or neurons cultured in medium from which the vitamin is absent (16). In vivo, cerebral ascorbate concentration is controlled by secretory and transport mechanisms of brain cells (15). Type-1 astrocytes are an abundant glial cell type comprising approximately 30% of brain volume. We showed previously that type-1 astrocytes from both rat and mouse brains possess a concentrative L-ascorbate uptake mechanism. Initial rate of ascorbate uptake saturated with increasing external ascorbate concentration, reflecting a high affinity interaction that could be described by Michaelis-Menten kinetics (17). Uptake was also dependent on external Na^+ concentration (17) and was inhibited by nonpermeant anion transport inhibitors (18), indicating that the ascorbate transporter is located in the plasma membrane. Preincubation with ouabain inhibited the initial rate of ascorbate uptake (17). Since ouabain inhibits the Na^+, K^+ ATPase that maintains transmembrane Na^+ and K^+ gradients, a possible explanation for this observation is that ouabain reduces the electrical potential across the membrane which, if the stoichiometry is more than

[1] Department of Physiology and [2]Division of Oral Biology, Faculty of Dentistry, University of Western Ontario, London, Ontario, Canada N6A 5C1.

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one Na^+ per ascorbate, would lead to inhibition of ascorbate influx.

A recent study of bovine pigmented ciliary epithelial cells indicated the presence of a membrane transport system in which two or more Na^+ were cotransported with each molecule of ascorbate (19). This study suggested that the high level of ascorbate in the aqueous humor of the eye is attributable to electrogenic Na^+-ascorbate cotransport across the ciliary epithelium. Furthermore, incubation with 200 μM ascorbate resulted in depolarization of the membrane potential by 4.9 mV (19).

It is important to characterize the transport process of astrocytes in order to understand the mechanisms underlying regulation of cerebral ascorbate concentration. The purpose of the present experiments was to determine if astroglial ascorbate transport activity is electrogenic and regulated by agents which alter membrane potential. Firstly, a kinetic analysis of the relationship between Na^+ and ascorbate uptake was carried out. Secondly, alteration of external K^+ and addition of ionophores were used to study the effects on ascorbate uptake of agents known to alter membrane potential.

EXPERIMENTAL PROCEDURE

Materials. L-[1-^{14}C]ascorbate (10 mCi/mmol) was purchased from Dupont Canada. Horse serum was obtained from Gibco Laboratories. Modified Eagle's minimum essential medium (MEM) (20) was made using tissue culture-grade chemicals purchased from Sigma. L-ascorbate, N(6),O(2′)-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP), gramicidin, DL-homocysteine, N-methyl-D-glucamine (NMG^+), nystatin (5880 USP units/mg) and valinomycin also were from Sigma.

Cells. Primary cultures of astrocytes were prepared from the neopallium of 1-day-old Sprague-Dawley rats according to the procedure of Hertz et al. (20,21). The cells were plated onto 60 mm petri dishes (Falcon) and grown in MEM with 20% horse serum (37°C; 95% air/5% CO_2). The culture medium was changed twice weekly, with the serum concentration reduced to 10% after 3 days. Cultures reached confluency after 2–3 weeks. They were grown for an additional 2 weeks, in the presence or absence of 0.25 mM dibutyryl cyclic AMP, before being used for uptake experiments. The cultivated cells stained positively for glial fibrillary acidic protein (GFAP) using the procedure of Wilson et al. (22). Treatment with 0.25 mM dibutyryl cyclic AMP changed the flat polygonal astrocytes to process-bearing stellate astrocytes and increased GFAP mRNA levels (23).

Measurement of Ascorbate Uptake. The initial rate of cellular uptake of L-[^{14}C]ascorbate was measured at 37°C in serum-free incubation media, essentially as described previously (17). The uptake medium for control experiments consisted of (in mM): 134 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 10 glucose and 20 HEPES, adjusted to pH 7.3 with NaOH. The role of external Ca^{2+} concentration was investigated by incubating cells in nominally Ca^{2+}-free uptake medium supplemented with 0.5 mM EGTA. To examine the effect of external Na^+ concentration on ascorbate uptake, Na^+ was isoosmotically replaced by NMG^+. To examine the effect of extracellular K^+ concentration, KCl was added to the Na^+-replete uptake medium. KCl was added to the medium, instead of substituting K^+ for Na^+, so as not to alter external Na^+ concentration. The effect of increased osmolality was assessed in parallel experiments in which equivalent concentrations of NMG^+ chloride were added to the Na^+-replete uptake medium containing 5.4 mM K^+. Stock solutions of L-[^{14}C]ascorbate and unlabeled ascorbate contained 0.4 mM homocysteine to prevent oxidation. The pH of the medium was not altered by the presence of these reductants at the concentrations employed. Aliquots of medium were collected at the end of each uptake incubation. Incubations were terminated by washing cultures with ice-cold, isoosmotic Tris-sucrose solution and harvesting the cells. An aliquot of the cell harvest was used for protein measurement (24) and the remainder was combined with scintillation cocktail. The radioactive contents of the medium and cells were measured by liquid scintillation counting.

Ascorbate uptake proceeds linearly with time for at least 3 min (17), so 1 min incubations were used to measure initial uptake rates. Rates were computed based on the specific activity of L-[^{14}C]ascorbate in the media and expressed as nmol ascorbate/g cell protein/min.

Statistics. Results are presented as the mean ± SEM of n experiments each with triplicate replications. The dependence of the initial rate of ascorbate uptake on external Na^+ concentration (S) was analyzed by Hill plots (25). The apparent maximum rates of ascorbate uptake (V_{max}) were obtained from Eadie-Hofstee and Lineweaver-Burk plots that were modified by substituting S^2 for S. For each plot, straight lines were fitted and intercepts calculated by linear regression. Comparisons between mean values based on a single level of treatment (e.g. initial rate of ascorbate uptake by cells incubated with or without external Ca^{2+}) were evaluated using paired *t*-tests. For simultaneous comparisons of two or more treatments, differences between means were evaluated using repeated measures analysis of variance and the Tukey-Kramer test (26). For all statistical tests, a P value of <0.05 was considered significant.

RESULTS

Ascorbate uptake proceeded more quickly in stellate than polygonal astrocytes. However, qualitatively similar effects of Ca^{2+}, Na^+, K^+, and ionophores were obtained whether or not astrocytes were pretreated with dibutyryl cyclic AMP to change their morphology from polygonal to stellate cells. The initial rates of ascorbate uptake were diminished by lowering the external concentrations of either Ca^+ or Na^+. Approximately 30% of initial ascorbate uptake was inhibited under conditions of low external free Ca^{2+} (Table I). In contrast, removal of external Na^+ inhibited the initial rate of ascorbate uptake by approximately 90% (Table I). Kinetic analysis of the relationship between Na^+ concentration and the rate of ascorbate uptake was carried out. Estimates of maximal uptake velocity (V_{max}) in the presence of 5 μM external ascorbate were obtained from two linearizations of the data. Modified Eadie-Hofstee plots (V/S^2 versus V) revealed straight lines with apparent V_{max} of 137 ± 22 and 229 ± 51 nmol ascorbate/g protein/min for polygonal

Table I. Effects of External Ca^{2+} and Na^+ on Initial Rates of Ascorbate Uptake[a]

	Polygonal	Stellate
Control	102 ± 11 (4)	150 ± 23 (4)
Ca^{2+}-free medium	65 ± 6 (4)*	107 ± 15 (4)
Control	123 ± 7 (4)	179 ± 15 (3)
Na^+-free medium	12 ± 4 (4)*	24 ± 6 (3)*

[a]Polygonal (not pretreated with dibutyrl cyclic AMP) and stellate (pretreated with dibutyrl cyclic AMP) astrocytes were incubated with 5 μM L-[^{14}C]ascorbate for 1 min at 37°C. The control incubation medium contained 138 mM Na^+ and 1.8 mM Ca^{2+}. Na^+ was replaced by NMG^+ in the nominally Na^+-free medium. The Ca^{2+}-free medium was nominally Ca^{2+}-free and supplemented with 0.5 mM EGTA. Values are the means ± SEM of n independent experiments performed in triplicate, and n is presented in parentheses. *$P < 0.05$ compared to control.

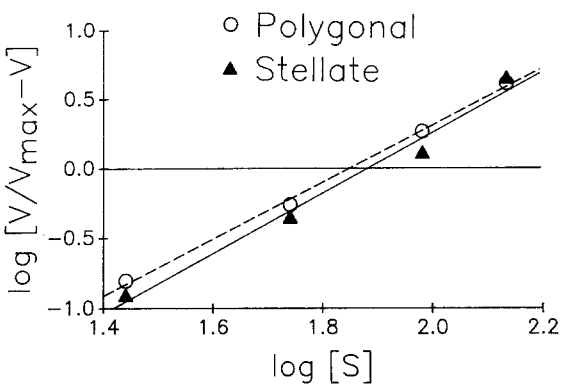

Fig. 1. Hill plots indicating the stoichiometry of Na^+-dependent ascorbate uptake. Polygonal (not pretreated with dibutyryl cyclic AMP) and stellate (pretreated 2 weeks with 0.25 mM dibutyryl cyclic AMP) astrocytes were incubated with 5 μM L-[^{14}C]ascorbate for 1 min at 37°C in media containing 5.4 mM K^+ and varied external Na^+ (N-methyl-D-glucamine$^+$ isoomotically substituted for Na^+). The initial rate of Na^+-dependent uptake (V) at each concentration of external Na^+ (S) was calculated as the difference between the total rate of uptake and the rate of Na^+-independent uptake (polygonal astrocytes, 14 ± 2 nmol ascorbate/g protein/min; stellate astrocytes, 25 ± 6 nmol ascorbate/g protein/min). Each point is the mean of three experiments performed in triplicate. Hill plots are based on apparent V_{max} of 137 and 229 nmol ascorbate/g protein/min for polygonal and stellate astrocytes, respectively, and yield Hill coefficients of 2.0 for polygonal astrocytes ($r^2 = 0.999$) and 2.1 for stellate astrocytes ($r^2 = 0.991$).

and stellate astrocytes, respectively. Similar results were obtained using modified Lineweaver-Burk plots (1/V versus $1/S^2$; data not shown). Hill plots based on the V_{max} estimates obtained from the V/S^2 versus V transformation of the data yielded Hill coefficients of 2.0 for polygonal astrocytes ($r^2 = 0.999$) and 2.1 for stellate astrocytes ($r^2 = 0.991$, Figure 1).

Our previous study (17) of the dependence of the initial rate of astroglial ascorbate uptake on external ascorbate concentration indicated a high affinity interaction that could be described by Michaelis-Menten kinetics, consistent with translocation of a single ascorbate anion per transport cycle. In the present study, we provide evidence that Na^+ and ascorbate are cotransported with a stoichiometry of greater than one Na^+ per ascorbate anion, predicting an electrogenic transport activity (Figure 1). To test this possibility, the effects on ascorbate uptake of agents known to alter membrane potential were examined.

Raising external K^+ incrementally from 5.4 to 100 mM, so as to depolarize the plasma membrane, immediately decreased the initial rate of ascorbate uptake, with the degree of inhibition depending on the concentration of K^+ (Figure 2). In contrast, control addition of NMG^+ chloride to the medium, to increase osmolality without increasing K^+ concentration, did not affect ascorbate uptake. Uptake rates in medium containing 138 mM Na^+ and 5.4 mM K^+ were 90 ± 13 and 168 ± 35 nmol/g protein/min in polygonal and stellate astrocytes, respectively, and adding 95 mM NMG^+ chloride to this medium yielded ascorbate uptake rates of 91 ± 10 and 142 ± 28 nmol/g protein/min in polygonal and stellate astrocytes, respectively (mean ± SEM of four independent experiments performed in triplicate).

When applied to cells, gramicidin and nystatin form channels through which monovalent cations flow (27), instantaneously collapsing membrane potential to zero

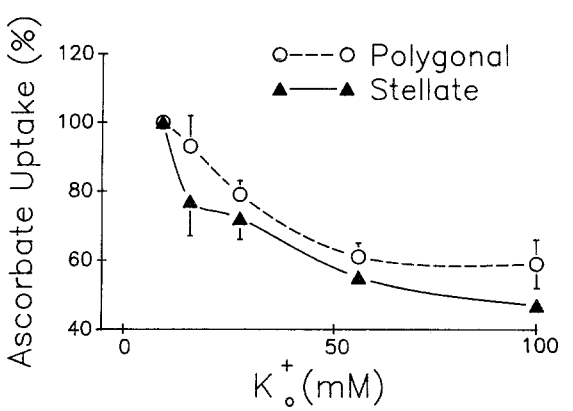

Fig. 2. Ascorbate uptake as a function of external K^+ (K^+_o). Polygonal astrocytes (cells not pretreated with dibutyryl cyclic AMP) and stellate astrocytes (cells pretreated with 0.25 mM dibutyryl cyclic AMP for 2 weeks) were incubated with 5 μM L-[^{14}C]ascorbate for 1 min at 37°C in media containing 138 mM Na^+ and varied K^+_o. Each point is the mean ± SEM of four experiments performed in triplicate. Results are normalized to uptake rates in medium containing 138 mM Na^+ and 5.4 mM K^+, which were 100% = 90 ± 13 and 100% = 168 ± 35 nmol ascorbate/g protein/min in polygonal and stellate astrocytes, respectively.

(28). In contrast, valinomycin is a highly selective carrier for K^+ (27), clamping membrane potential at the K^+ equilibrium potential (28). We observed that the depolarizing ionophores gramicidin and nystatin immediately slowed ascorbate uptake, whereas the nondepolarizing ionophore valinomycin had no detectable effect (Figure 3). Inhibition by the depolarizing ionophores did not require preincubation, since either gramicidin or nystatin was added simultaneously with L-[^{14}C]ascorbate at the beginning of the 1 min uptake incubation.

DISCUSSION

The Na^+-dependence of astroglial ascorbate uptake gave Hill coefficients of approximately two (Fig 1), suggesting a stoichiometry of two Na^+ for each ascorbate anion translocated across the plasma membrane. However, the Hill coefficient may reflect other factors (19,25) and a stoichiometry other than 2:1 cannot be ruled out. In previous studies of Na^+-coupled ascorbate uptake, Helbig et al. (19) reported a Hill coefficient of 1.94 for bovine ciliary epithelial cells whereas Diliberto et al. (29) reported coefficients nearer to unity (1.1 with 25 μM ascorbate and 1.4 with 200 μM ascorbate in the medium) for bovine adrenomedullary cells. Another respect in which ascorbate transport by astrocytes and adrenomedullary cells differs is that lowering external Na^+ concentration decreases the apparent V_{max} of ascorbate uptake in astrocytes (17) but not in adrenomedullary cells (29).

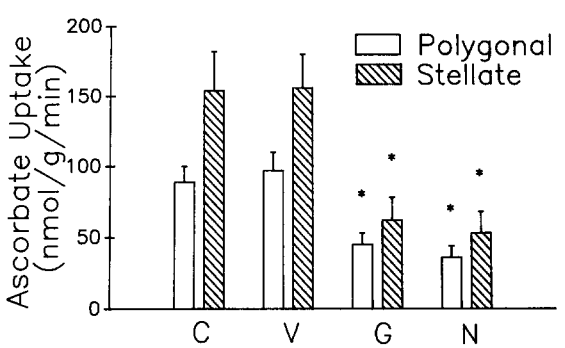

Fig. 3. Acute effects of ionophores on initial rates of ascorbate uptake. Polygonal (not pretreated with dibutyryl cyclic AMP) and stellate (pretreated with dibutyryl cyclic AMP) astrocytes were incubated with 5 μM L-[^{14}C]ascorbate for 1 min at 37°C in medium containing 5.4 mM K^+ and 138 mM Na^+. Valinomycin (V, 1.5 μM), gramicidin (G, 1 μM) or nystatin (N, 100 mg/l) were added to the medium simultaneously with L-[^{14}C]ascorbate. Controls (C) received vehicle. Each point is the mean ± SEM of five experiments performed in triplicate. *$P < 0.05$ compared to control.

Using bovine adrenomedullary cells, Diliberto et al. (29) observed that simultaneously lowering external Na^+ and raising K^+ concentrations decreased ascorbate uptake. However, this effect was attibuted mostly to Na^+ substitution. In the present experiments, KCl was added to the medium, instead of substituting K^+ for Na^+, so as not to disturb external Na^+ concentration. The effect of increased osmolality on ascorbate uptake was found to be neglible when assessed by addition of NMG^+ chloride to the medium. The high permeability of astrocytes to K^+, however, renders their membrane potential virtually a K^+ equilibrium potential and sensitive, therefore, to alterations in the external concentration of this cation (30,31). In situ, K^+ released by discharging neurons may act as a signal to adjacent astrocytes and selectively alter certain transport activities (31,32). Those transport activities that are sensitive to K^+ may be identified through studies of cultured astrocytes, which already have shown that raising K^+ to 20 mM increases 2-deoxyglucose uptake (33,34) but has no effect on sorbitol uptake (35). The present experiments indicate that high K^+ inhibits the rate of clearance of ascorbate from the extracellular fluid into astrocytes (Figure 2). Periods of neuronal firing activity such as occur during seizures can elevate extracellular K^+ to 10–12 mM (36–38) and may thereby alter astroglial ascorbate transport.

The membrane potential of stellate astrocytes has been observed to change by 55–57 mV per 10-fold change in external K^+ at concentrations greater than 10–20 mM (K^+ was substituted for Na^+; 39). Based on those observations, it would be predicted that 55 mM K^+ would depolarize the membrane to about −20 mV and 100 mM K^+ to about −5 mV. Therefore, the electrical gradient favoring Na^+-ascorbate uptake would be largely dissipated at 55–100 mM external K^+ concentration. We found that, under these conditions, the initial rate of ascorbate uptake was decreased by approximately one-half (Figure 2). Similarly, abolition of the membrane potential by nystatin or gramicidin (28), reduced the initial rate of ascorbate uptake by approximately one-half (Figure 3). It may be concluded that membrane potential contributes significantly to the mechanism that mediates concentrative Na^+-ascorbate uptake by astrocytes.

In contrast to the effects of gramicidin and nystatin, addition of the K^+ ionophore, valinomycin, did not change the velocity of ascorbate uptake in astrocytes (Figure 3). The resting membrane potential of astrocytes (−75 mV; 40) is so close to the equilibrium potential for K^+ that any hyperpolarizing effect of the K^+ ionophore (28) was

likely too small to cause a detectable increase in the initial rate of ascorbate uptake.

The dependence of ascorbate transport on membrane potential may provide a physiologoical mechanism for regulation of vitamin C availability in astrocytes. Astroglial cells possess receptors for neurotransmitters and activation of these receptors may, in many cases, alter membrane potential (28,40–42) and thus influence the transmembrane movement of ascorbate.

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